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14. ABSTRACT The purpose of this study is to combine the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat metastatic breast cancer. This is based on the rationale that RT can be limited to 1 or 2 lesions which in turn serve as antigenic reservoirs that drive abscopal effects. Patients receive either 1mg or 10mg of the anti-TGFbeta antibody.

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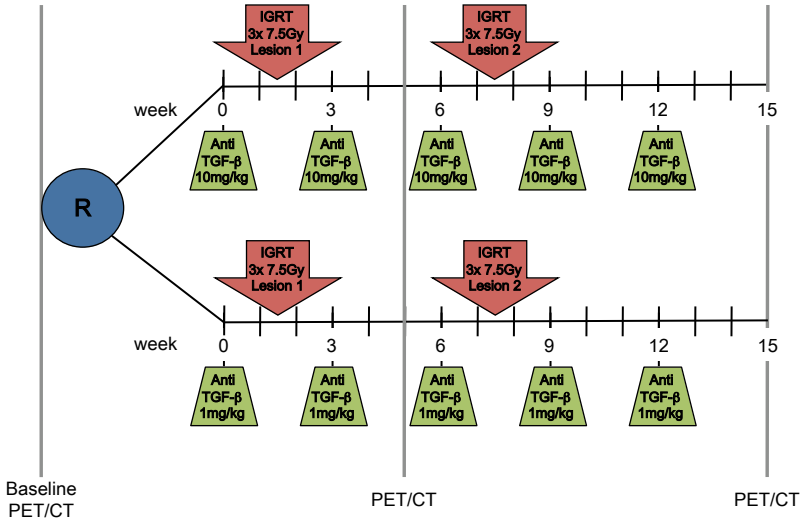
1 Introduction

The purpose of this study is to combine the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat metastatic breast cancer. This is based on the rationale that RT can be limited to 1 or 2 lesions which in turn serve as antigenic reservoirs that drive abscopal effects. Patients receive either 1mg or 10mg of the anti-TGFbeta antibody. The primary objectives are to assess safety, feasibility and tumor regression and to monitor immune responses in these patients. The abscopal effects are assessed by imaging. The UCLA component is 3 fold. 1) to enroll patients into the clinical trial 2) to assess immune responses using blood samples before, during and after treatment by multi-channel flow cytometry for immune monitoring 3) to examine the effects of Fresolimumab on breast cancer stem cells.



2 Body

2.1 Immune-monitoring detecting overall trends

The collection of samples from the final cohort of patients has been completed. These are in the process of being analyzed together to allow better comparison of responses. In the meantime, we have focussed on finding pattern of responses and optimizing the presentation of data. This has involved rigorous rechecking of all samples and reanalysis. There is a growing awareness in the field that relative changes can be most relevant as the immune system is essentially in a balance of forces. Evaluation of any one response without considering it against others can therefore be misleading. This is a relatively new area of research and there are no hard guidelines, but we are developing these as we go along. In a sense, this analysis can be thought of as having an in-built control monitor that adds rigor to any analysis.

The most basic ratio of CD4:CD8 lymphocytes has been used for a long time as a reflection of immunological responses. Fig 1 shows the data from the trial. By week 2, which is right after the first dose of Fresolimumab and radiation therapy (RT), there is an increase in this ratio, clearly indicating a direct effect of the combination treatment, and it seems largely due to a changes

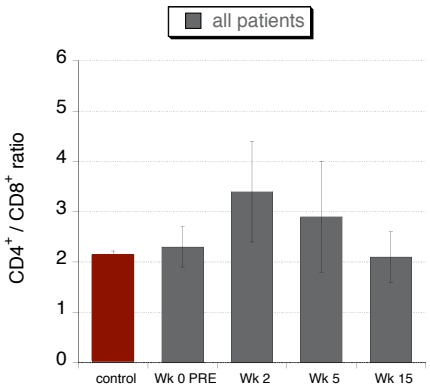


Fig 1: CD4:CD8 ratio changes

in CD4. In fact, CD4 T cells markedly increase in most patients on week 2 (not shown), especially the Th1 subset (Fig 2). Whereas CD8 numbers as a % of all lymphocytes vary little with treatment time, although they differ from 4-40% between individual patients (not shown).

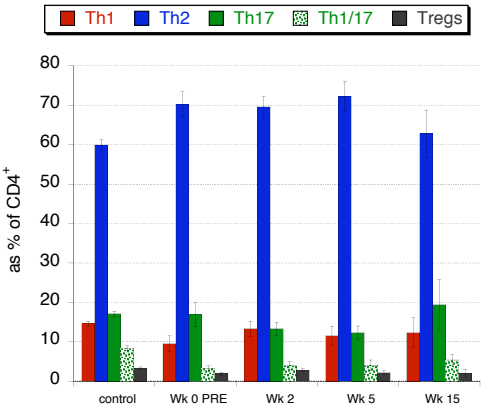


Figure 2: CD4 T cell subsets

Importantly, a plot of Th1:Th2 clearly shows an immediate and lasting treatment-induced rise towards normalcy (Fig 3). Within the T helper compartment there are several other subsets of interest, particularly T regulatory cells because they suppress responses. While Tregs levels are frequently reported as being high in many cancer patients, this was not the case for most of our advanced breast cancer patients (not shown). As a % of total lymphocytes, Tregs did increase following combination treatment, but this is in fact misleading because the ratio of Th1:Tregs actually changed in favor of Th1 cells (Fig 4), indicating a favorable effect on the immune system perhaps allowing the activation of anti-tumor responses. However, this was not entirely supported by the finding that the ratio to all-important CD8+ cells fell in week 2 and 5 before returning to pre-treatment levels by the end of the treatment course (Fig 5). Such discrepancies can arise from differences in the response kinetics for these populations. Since Tregs can be converted into Th17 cells in a TGF-beta dependent manner, we examined the ratio of Tregs: Th17 cells, which increased suggesting that the changes seen in Tregs were not due to conversion. In fact, it suggests that anti-TGF-beta treatment may have prevented such conversion as might be expected.

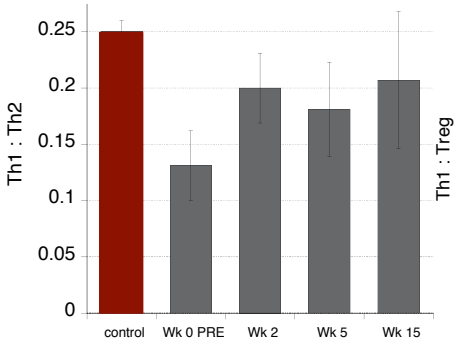


Figure 3: Th1:Th2 ratios

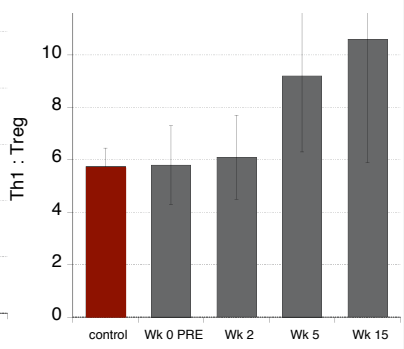


Figure 4: Th1:Treg ratios

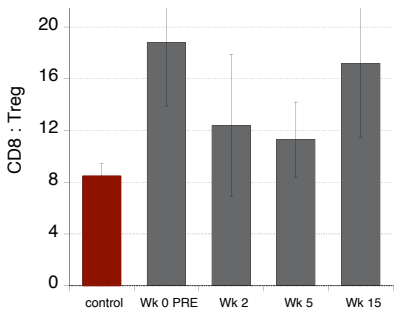


Figure 5: CD8:Treg ratios

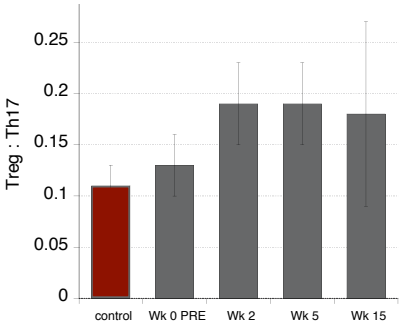


Figure 6: Treg:Th17 ratios

These reanalyses have uncovered some important points about treatment with Fresolimumab that might have been missed at first glance and stress the importance of spending the considerable amount of time required to perform a complete analysis of the data.

2.2 Immune-monitoring that tracks individual responses

Important statistical input has also revealed in the meantime that women receiving 10mg had a longer survival than those receiving 1mg of drug. It was therefore crucial to reanalyze and stratify the immune monitoring data according to the dose of Fresolimumab. And this is presented here.

CD8+ T cells specific for the tumor antigen survivin were analyzed in HLA-A2.1+ patients under the assumption that high or rising levels of these cells point to an active anti-tumor immune response. Indeed, significant levels pre-existed in 3 of 8 patients (N01 was a repeat and gave the same value both times, adding validity to the assay) (Fig 7). All 3 with high pre-treatment levels received the 1 mg dose and only 1 of these (N05) responded to treatment. Of the 5 that had low preexisting levels, 3 increased with time (N02, U03, and U07 marginally); all received 10mg doses. In summary, 3 of 4 receiving 10mg doses showed increased reactivity with time compared to only 1 of 4 at 1mg doses. Of the 4 HLA-A2.1+ patients who completed the course till 15 weeks, 3 were on 10mg doses and had increased tumor-specific responses. The 4th, who received 1mg, had high pre-treatment levels that did not change. This analysis is encouraging, although the numbers are very small.

CD4+ Treg cells were at normal levels in almost all patients except N05, who was high. The very same patient (N05) also had the highest pre-existing levels of tumor-specific T cells (Fig 7). Overall Treg levels tended to increase after the first round of treatment (at 2 weeks) in patients receiving 10mg, but not 1 mg (Fig 8), and they continued to rise in 2 patients (U03, N02), both of whom received 10mg doses and responded in the tetramer assay. In these 2 patients the CD8: Treg ratio fell with time (not shown). Of the 7 patients who stayed the course until week 15, five had received the 10mg dose.

In summary, Treg levels were not particularly abnormal in these cohorts and tended to track with responsiveness in the tetramer assay, which reflects the natural tendency of Tregs to mirror changes in the CD8+ T cell compartment presumably to counteract immune activation in order to limit tissue damage.

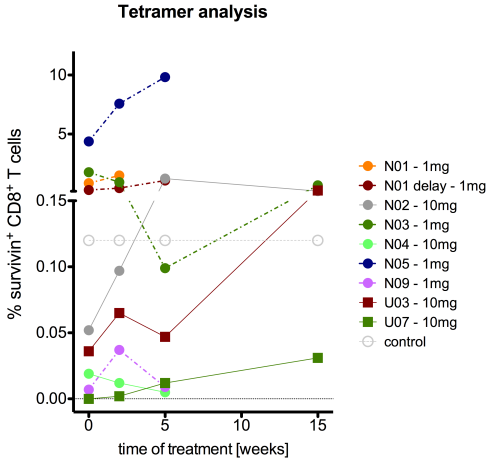


Figure 7: tetramer analysis of tumor-specific CD8+ T cells.

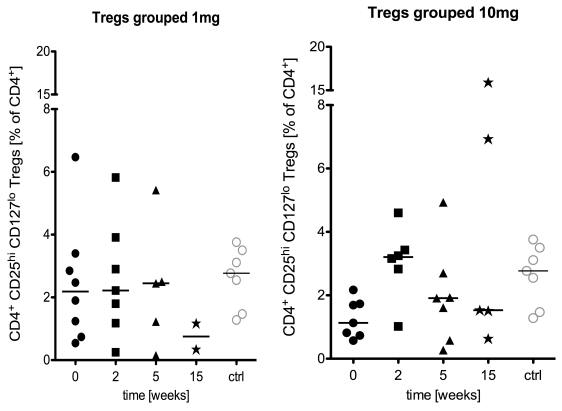
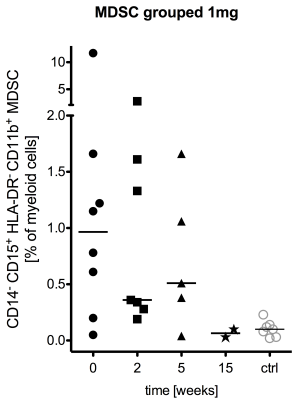


Figure 8: Treg responses – grouped data

White cell counts were low in many patients (Fig 10) and increased in those receiving 10mg, but not 1mg of drug. In general, Th1 cell counts tended to be on the low side, whereas Th2 cells, which in general were higher than normal prior to treatment in all patients, had the tendency to shift to normal values following treatment (not shown). In other words, Th1:Th2 ratios normalised during treatment, but with little dose dependency.

In the myeloid compartment, inflammatory monocyte numbers varied considerably prior to treatment, but tended to increase to normal levels after doses of 10mg (not shown). CD14-CD15+HLA-DR-CD11b+ cells -phenotypically considered as belonging to the myeloid derived suppressor cell compartment- were elevated in many patients before treatment and fell substantially with time in most (Fig 9).



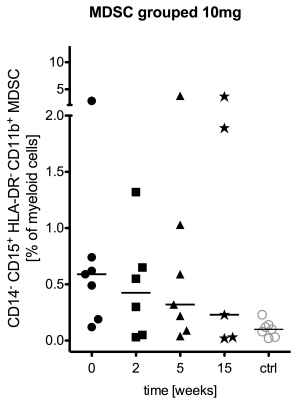
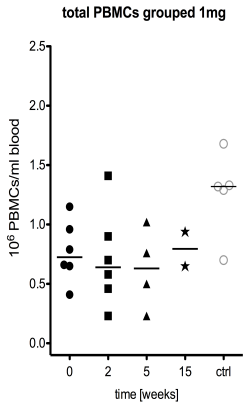


Figure 9: Myeloid suppressor cells



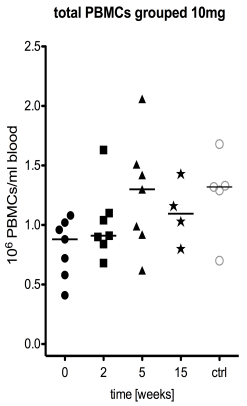


Figure 10: PBMC counts

We also screened patient sera for antibody responses against a panel of 34 common tumor antigens (Fig 11) in an attempt to assess the involvement of the B cell compartment in our patient population. Four patients had pre-existing responses to >5 of these antigens. Two of these patients significantly decreased their tumor-specific antibody load with time, both had gotten 1mg of drug plus RT. Of the remaining 11 patients who started with low antibody reactivity, 3 increased in responsiveness to a score of >1 and all 3 had received 10mg doses.

The 2 patients who converted to survivin-specific responses in the tetramer analysis (U03 and N02) also had rising survivin antibody titres (Fig 12), while another (N03) had both high pre-existing survivin-specific T cells and pre-existing survivin-reactive antibodies, although a broad correlation between anti-survivin T cell and B cell responses was not present (Fig 12).

In summary, the immune monitoring shows more favorable responses to 10 mg than 1 mg, that is consistent with the improved

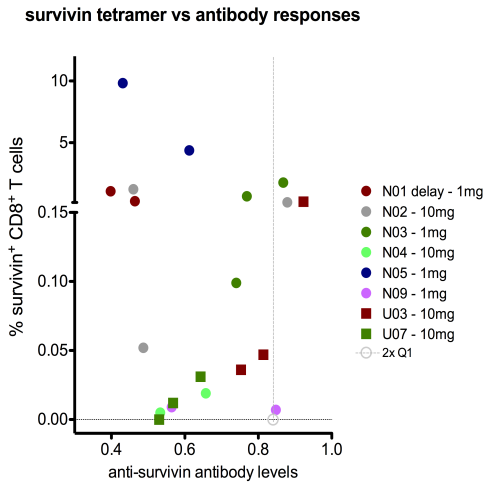
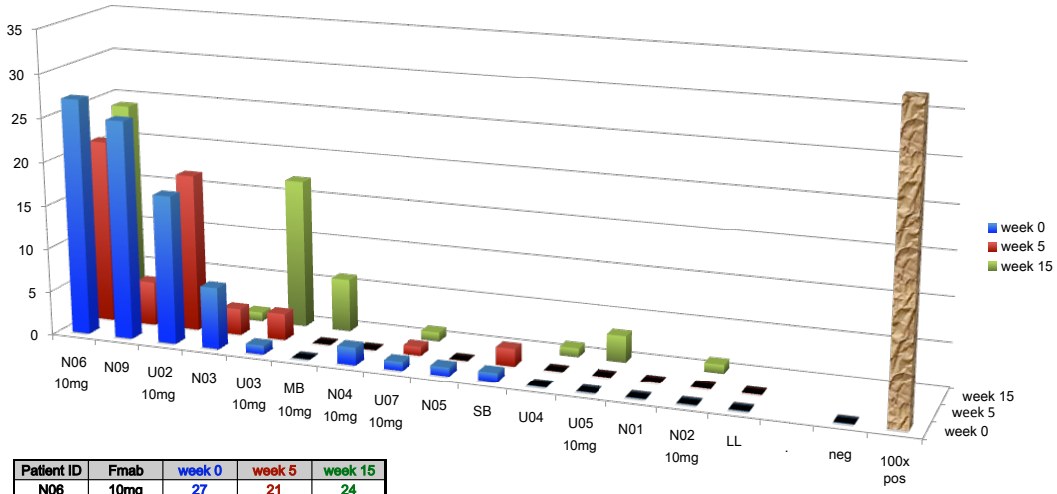


Figure 12: Correlation between anti-survivin T cell and antibody responses.

survival of this cohort of patients.

Figure 11: Antibody responses

combined antibody score
CABYR
CSAG2
CTAG1B
CTAG2
CYCLINB1
CYCLIND1
GAGE1
HER2
HSPA4
HSPD1
HTERT
LDHC
MAGEA1
MAGEA3
MAGEB6
MICA
MUC1
MYCPB
NLRP4
P53
PBK
PRAME
SILV
SPANXA1
SSX2
SSX4
SSX5
SURVIVIN
TRIP4
TSSK6
TULP2
WT-1
XAGE
ZNF165



Patient ID	Fmab	week 0	week 5	week 15
N06	10mg	27	21	24
N09	1mg	25	5	
U02	10mg	17	18	
N03	1mg	7	3	1
U03	10mg	1	3	17
MB	10mg	0	0	6
N04	10mg	2	0	
U07	10mg	1	1	1
N05	1mg	1	0	
SB	1mg	1	2	
U04	1mg	0	0	1
U05	10mg	0	0	3
N01	1mg	0	0	
N02	10mg	0	0	1
LL	1mg	0	0	

2.3 Immune-monitoring finding pattern of responses

Our most recent efforts aimed at capitalizing on the extensive amount of immune monitoring data that we have accumulated from this small patient cohort and to further tease out Fresolimumab dose- and time- dependencies in order to maximize our understanding of immunological response patterns and its relation to outcome.

Two of the most promising responders (based on antibody spread and/or time to progression), namely U03 and U07, who received the higher, 10mg dose of the antibody were stratified according to overall trends of their individual immune parameters. A lot of these parameters co-track over time, i.e. overall upward or downward (Fig 13, U03, not shown U07). Surprisingly, considering the complexity and individuality of the immune system, many of these tracks overlap between both patients. Of these, perhaps the most intriguing, common trends in terms of anti-tumor immune responses were the overall rise in tumor-specific CD8+ T cells, Th1/Th2 and NK cells in contrast to Th2 cells, MDSCs, mDCs, CD4+ cells and CD8/Treg ratio that were falling.

Whether such patterns of responses are meaningful or have any prognostic value is too early to say. Clearly, extrapolating from such a small data set needs

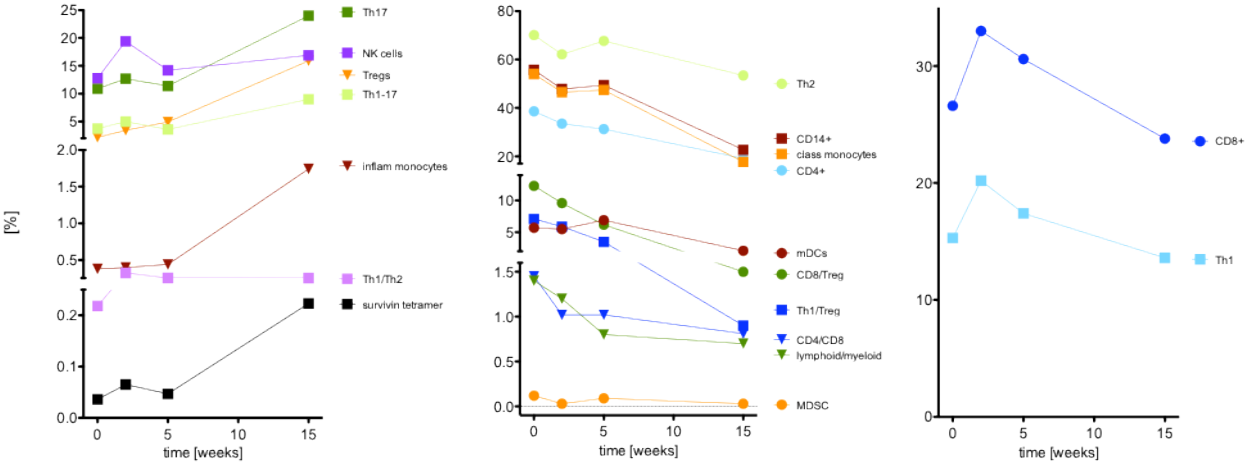


Figure 13: Individual responses for Patient U03

extreme caution. However, it is encouraging that these immune fluctuations indeed appear to reflect changes seen in other patients as well, as we alluded to in our last report.

In an attempt to better grasp therapy-related changes for both cohorts without losing sight of individual responses we illustrated some of the most promising immune parameters in waterfall plots. This was further stratified into an early phase (changes from week 0 to week 2) and a late phase (week 0 to week 15), which may allow us to appreciate possible time-dependencies. Naturally, the late phase data set is less powerful since not every patient completed the full treatment course. This becomes even more limiting for tumor-targeting survivin-specific CD8+ T cells due to the HLA-restricted tetramer assay. Changes in these highly specific immune players remain relatively small within the first 2 weeks (Figure 14), which is not unexpected considering the known delayed nature of the response. As pointed out in our previous report, of the 4 HLA-A2.1+ patients who completed the course till 15 weeks, 3 (on 10mg doses) had increased tumor-specific responses. The 4th, who received 1mg, had high levels to begin

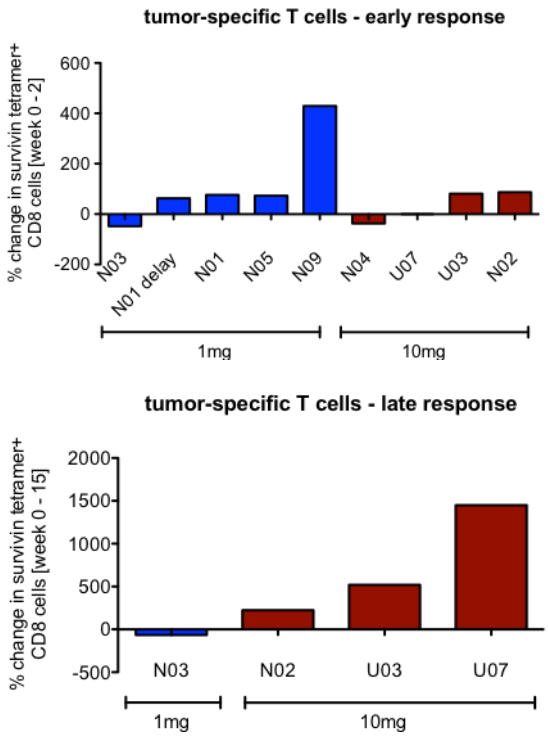


Figure 14: Waterfall plots for survivin-specific CD8+ T cells changes during week 0-2 (top) and week 0-15 (bottom).

with that did not change.

When looking at some of the other immunological factors one can immediately appreciate that the representation of patients from the two treatment arms changes drastically from a 50/50 divide at week 2 (6 patients each from the 1mg Fresolimumab arm [blue] and from the 10mg-arm [red]) to a 30/70 ratio at week 15 with most of the high-dose group completing the 15-week treatment whereas only 2 patients in the low-dose arm remained (Fig 15). Again, this illustrates better survival in the 10mg-arm that came to light after statistical analysis (see NYU report).

Obvious is also the rise in Tregs in the 10mg group that we have noticed before and that persists over time (Fig 15). In fact, it mirrors the response of rising tumor-specific T cells (Fig 14), while the one patient of the 1mg-cohort we have this data for (N03) shows consistently lower levels for both Tregs and tumor-specific T cells than before treatment. It is perhaps not surprising then that all of the 10mg patients returned with falling ratios of CD8+ T cells to Tregs that was less obvious in the 1mg cohort (Fig 15).

For the most part these data confirm our previous findings and conclusions. Clearly, teasing out obvious patterns of responses from this small data set remains a challenge, one that the field as a whole is struggling to embrace and we will continue to address.

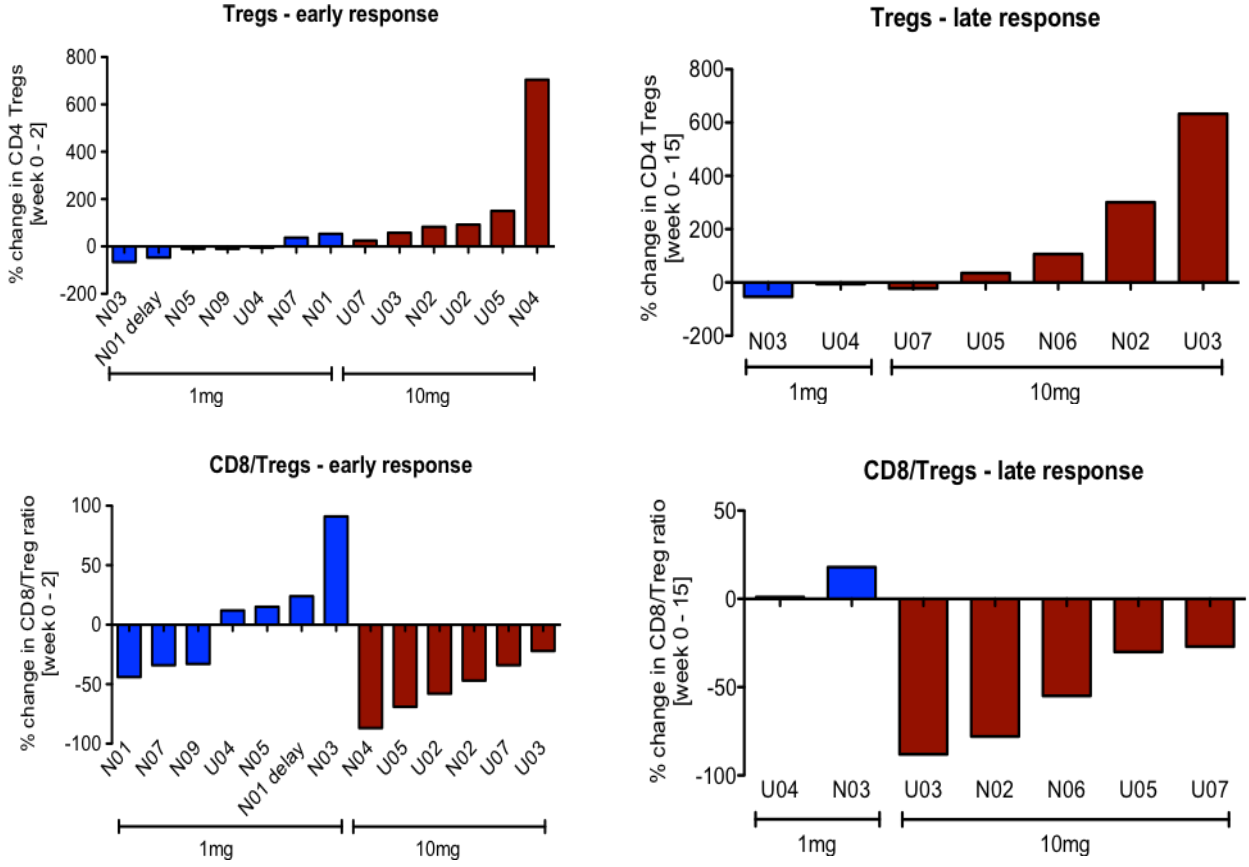


Figure 15: Waterfall plots illustrating changes in Tregs and in CD8/Tregs. Patients receiving 1mg Fresolimumab are shown in blue, those who got 10mg are in red. Early changes (week 0-2) are on the left and late changes (week 0-15) are on the right.

2.4 Monitoring the patient's general immune status

The kynurenine-to-tryptophan ratio in the circulation is indicative for indoleamine-pyrrole 2,3-dioxygenase (IDO) activity at the tumor site and may provide us with another measure of the ability of Fresolimumab + RT, namely whether a "dangerous" tumor microenvironment has been generated that might stimulate tumor immunity. Elevated IDO activity raises kynurenine while depleting tryptophan, both associated with immune suppression and chronic inflammation. TGF-beta is known to maintain an IDO-driven regulatory environment. It is therefore, reasonable to assume that by blocking TGF beta, we maybe able to disturb the TGF-beta-IDO axis and brake the self-sustaining immune supression. Indeed, we are excited to report that plasma from a subset of Fresolimumab + RT-treated patients displayed a marked decline in the kynurenine/tryptophan ratio (Fig 16), perhaps hinting at a reversal of immune suppression in these patients following the combination treatment.

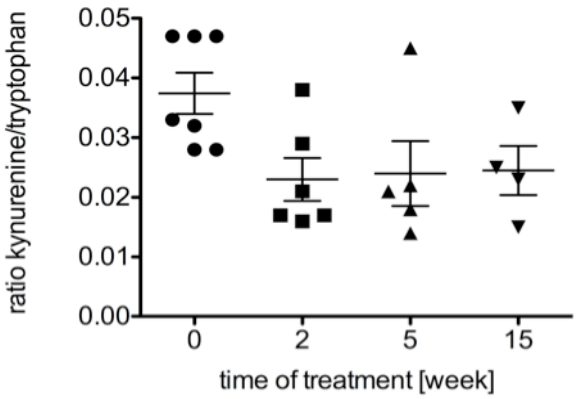


Figure 16: Kynurenine/Tryptophan ratio in plasma samples taken from the UCLA patients.

2.5 Cancer Stem Cell Response to TGF-beta inhibition

Recent preclinical and clinical data support that solid cancers including breast cancers, are organized hierarchically with a small population of cancer stem cells (CSCs), capable of re-growing the entire tumor while their progeny lack this ability. We, and others, reported that breast CSCs (BCSCs) are relatively resistant to ionizing radiation and after irradiation, the surviving BCSCs are recruited from a quiescent state (G0) into an active cell cycle, allowing repopulation of the tumor. Furthermore, we have shown that RT can induce reprogramming of non-tumorigenic cancer cells to generate new cancer stem cells (induced cancer stem cells, iCSCs). The mechanisms involved require the re-expression of the stem cell transcription factors Oct4, Sox2, Klf4 and Nanog. Interestingly, this re-expression was higher in polyploid cells.

TGFβ activation is a regulator of BCSC expansion and the project aims at investigating its effect on reprogramming of non-BCSCS into BCSCs. Interestingly, we have observed varying effects for BCSCs when breast cancer cells were treated with an inhibitor of the TGFβ receptor. We are currently investigating this divergence further. In order to do this in the manner most relevant to the clinical trial we are using Fresolimumab to dissect the role of TGF-beta in stem cell reprogramming after RT. This

is important if we are to further understand the effects of Fresolimumab and RT on BCSCs and this work is ongoing.

Previously, we reported that a single treatment with a selective inhibitor of TGF-β receptor (SB-431542) efficiently inhibited the spontaneous (Fig. 17A) and radiation-induced (Fig. 17B) reprogramming of the claudin-low breast cancer line, SUM159PT, based on the expression of our ZsGreen-cODC reporter for breast cancer stem cells. In order to assess the effect of this inhibitor on the self-renewal capacity of the reprogrammed cells, we followed up these experiments with functional assays (sphere forming capacity). These experiments indicate that while the inhibitor has a significant effect on the percentage of the radiation-induced ZsGreen-pos cells (a fluorescent reporter for BCSCs), it only has a small effect on their self-renewal capacity. These experiments need to be repeated with repeated doses of the inhibitor (5 daily treatments) and compare with the single treatment regimen. We then tried combining the inhibitor with different doses of radiation, including a fractionated regimen of 5x2Gy. These experiments showed that the effect of the inhibitor was greatest at the low dose of 4Gy, and when combined with fractionated radiation treatment (Fig. 17D)

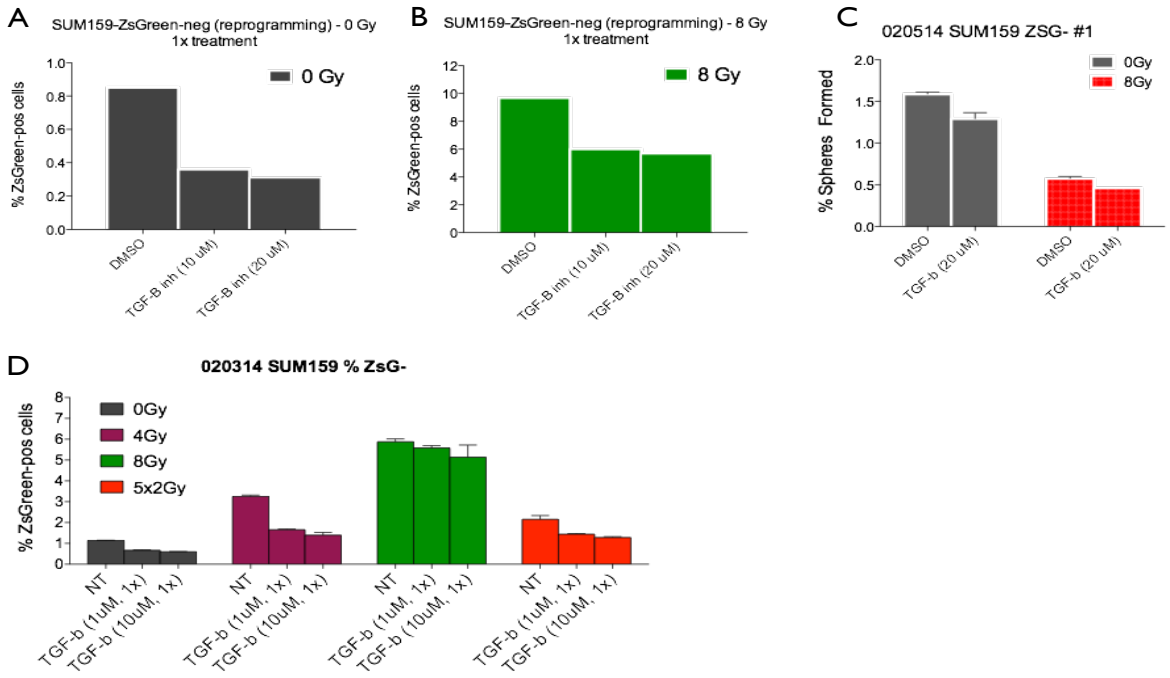


Figure 17: Effect of TGF-b inhibitor on the radiation-induced reprogramming of SUM159PT

The effect of the inhibitor on radiation-induced enrichment of breast cancer stem cells (BCSCs) in mammosphere cultures of MDA-MB-231-ZsGreen-cODC are shown in 2 representative experiments in Fig. 18A and B. Single doses of radiation enriched for BCSCs in a dose-dependent manner (Fig. 18B), and this effect was partially abrogated by a single treatment with 10uM of the TGF-β inhibitor 3 hours prior to irradiation. In contrast, the existing pool of BCSCs in MDA-MB-231 line, was depleted of stem cells only after 5 consecutive treatments with 1uM of the inhibitor (Fig. 18C).

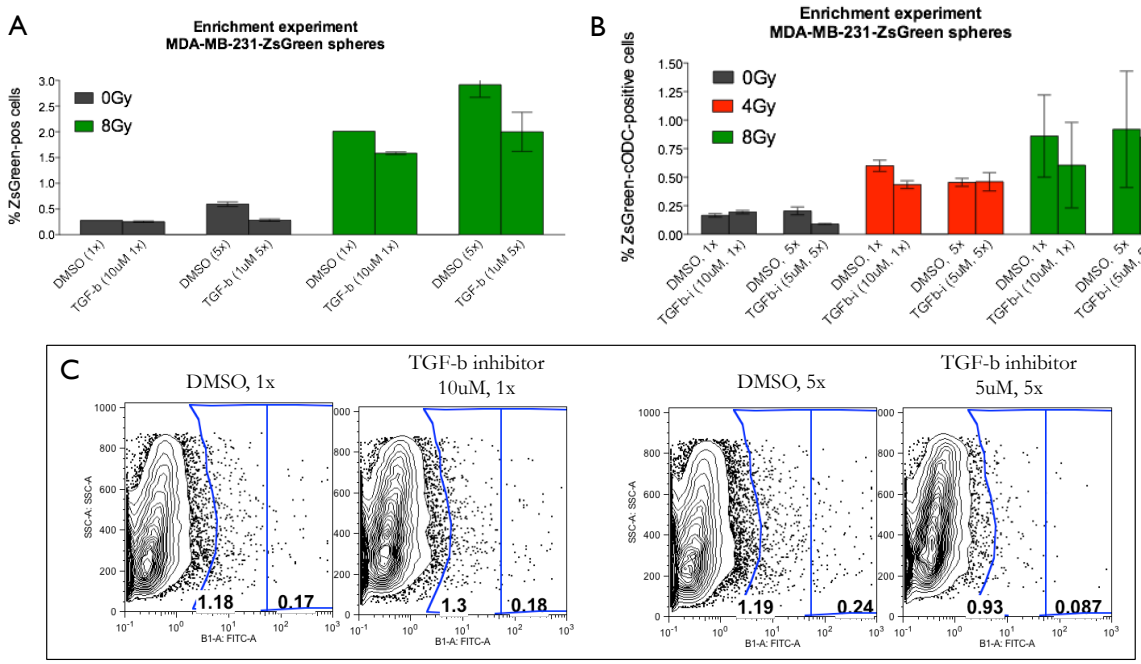


Figure 18: Effect of TGF-b inhibitor on the radiation-induced enrichment of CSCs in MDA-MB-231-ZsGreen-cODC

3. Problems Encountered

There was a slow down in subject enrollment and we took steps to correct this, though unfortunately the drug was removed from availability by the manufacturer and we were unable to enroll the last 6 patients due to expiry limits imposed on the reagent. Since then, two breast cancer clinicians and an additional research nurse have been recruited and we are in a strong position to continue the trial with a small molecule TGF-beta inhibitor. There have been no problems to report regarding the conduct of study procedures, consenting, confidentiality or anything else that would be considered reportable. All SAEs and AEs have been reported to NYU and the UCLA IRB as per protocol.

4. Future directions

The enrollment has been completed and we are in the final phase of immune monitoring and expect to be completed in the next period. Hopefully this will consolidate our previous findings and help us to tease out clear response patterns. We will be working closely with our statistician to bring the study to a successful conclusion and publish the findings. The anti-TGF-beta studies with cancer stem cells will continue to investigate the importance of this pathway in radiation-induced reprogramming.

5. Key Research Accomplishments

- Trial recruitment is complete
- Immune monitoring and extensive analysis thereof is yielding interesting data, especially at the 10mg dosage.
- TGF-beta affects non-stem cell reprogramming by radiation exposure in a manner that depends on the origin of the breast cancer cell line.
- Schaue et al. Radiation & Inflammation. Seminars in Radiation Oncology (in press)
- Vlashi et al. Metabolic differences in breast cancer stem cells and differentiated progeny. Breast Cancer Res Treat (in press)
- Vlashi et al. Cancer Stem Cells, Cancer Cell Plasticity and Radiation Therapy. Sem in Cancer Biol (in press)